Arms races with mitochondrial genome soft sweeps in a gynodioecious plant, Plantago lanceolata

PROF. DEBORAH CHARLESWORTH (Orcid ID : 0000-0002-3939-9122)

Article type : Original Article

Arms races with mitochondrial genome soft sweeps in a gynodioecious plant, *Plantago lanceolata*

Authors

R. Bergero [†], N. Levsen [‡], K. Wolff [‡], D. Charlesworth [†]

Author Affiliations:

[†] Institute of Evolutionary Biology, Ashworth Laboratory, School of Biological Sciences, The University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3FL, United Kingdom

[‡] School of Natural and Environmental Sciences, Newcastle University, Newcastle Upon Tyne, Tyne and Wear, NE1 7RU, United Kingdom

Corresponding Author: Deborah Charlesworth (Deborah.Charlesworth@ed.ac.uk)

ABSTRACT

Biological situations involving conflict can create arms race situations with repeated fixations of different functional variants, producing selective sweeps and lowering neutral diversity in genome regions linked to the functional locus. However, they can sometimes lead to balancing selection, potentially creating long coalescent times for sites with functionally different variants, and, if recombination occurs rarely, for extended haplotypes carrying such variants. We tested between these possibilities in a gynodioecious plant, *Plantago lanceolata*, in which cytoplasmic male-sterility factors conflict with nuclear restorers of male fertility. We find low mitochondrial diversity, which does not support very long-term coexistence of highly diverged mitochondrial haplotypes. Interestingly, however, we found a derived haplotype that is associated with male fertility in a restricted geographic region, and that has fixed differences from the ancestral sequence in several genes, suggesting that it did not arise very recently. Taken together, the results suggest arms race events that involved “soft" selective sweeps involving a moderately old-established haplotype, consistent with the frequency fluctuations predicted by theoretical models of gynodioecy.

Introduction

Gynodioecious populations of plants exemplify a form of intra-genomic conflict. When the organelle genomes are maternally transmitted through the egg cytoplasm in hermaphrodite populations, a cytoplasmic genetic factor causing loss of male functions (a cytoplasmic male-sterility mutation or CMS factor) is favoured by selection if it increases female reproductive fitness (Dufay & Billard, 2012; Lewis, 1941; McCauley & Olson, 2008). As females spread in the population, and hermaphrodite individuals become increasingly rare, the supply of pollen will eventually limit females’ seed production and can result in a balanced polymorphism for the CMS factor, if the population is self-compatible. If the hermaphrodites are self-incompatible, the pollen supply limits the seed production of hermaphrodites and females equally (Baker, 1963), and a CMS polymorphism cannot be maintained by this simple frequency dependence. Nevertheless, self-incompatible gynodioecious plant species are known (Baker, 1963; Ross, 1973), including *Plantago lanceolata*, which has females in most natural populations that have been surveyed, across a wide geographic range (Bartlett, 1911), with genetic control involving multiple polymorphic CMS factors (Van Damme, 1983; Van Damme & VanDelden, 1982). CMS polymorphisms can be maintained in these gynodioecious populations if they are also polymorphic for nuclear restorer alleles (Van Damme, 1986), producing joint polymorphisms under defined conditions, as reviewed by (Jacobs & Wade, 2003; Lahiani, Touzet, Billard, & Dufaÿ, 2015).i

Situations with joint cytoplasmic and nuclear control of male sterility produce allele frequency dynamics very similar to those for host-pathogen systems (Tellier & Brown, 2007), another situation involving conflict. Both cases have two possible outcomes, successive fixations of functionally different alleles in the two players (often called "arms races") or maintenance of polymorphism in both players (sometimes called "trench warfare"). In male-sterility systems with CMS and nuclear restorer alleles, single-population models show that successive fixation of new CMS factors may occur as nuclear restorers of male fertility arise (Frank, 1989). Alternatively, if the nuclear restorer alleles cause a fitness disadvantage, variability may be maintained for both factors, which may undergo long-term cyclical frequency changes (Couvet, Ronce, & Gliddon, 1998; Gouyon, Vichot, & Van Damme, 1991), or reach a point equilibrium frequency after damped cyclical changes that may last for prolonged time periods (Charlesworth, 1981; Delannay, Gouyon, & Valdeyron, 1981).

In a metapopulation, however, if females have a higher seed output than hermaphrodites, they found most new colonies. Couvet et al. (1998) studied a model of a self-incompatible species like the *P. lanceolata* populations studied here. With two CMS factors and dominant specific restorer alleles at two nuclear loci, joint polymorphism for nuclear and CMS factors could result, yielding low female frequencies, like those in gynodioecious *P. lanceolata* populations, provided that the population did not go extinct or lose the gender variation. Frequent deme extinctions, however, caused loss of the nuclear polymorphism, resulting in pure CMS. A metapopulation with a single CMS/non-sterility factor and a single nuclear restorer locus can also maintain a cytonuclear polymorphism under conditions where this would not occur in a single population. However, cytoplasmic polymorphism was never maintained within sub-populations, and was sometimes lost throughout the metapopulation, resulting in purely nuclear control of femaleness (Dufay & Pannell, 2010). Importantly, if the presence of females is due to metapopulation dynamics, bottlenecks must occur frequently, predicting that mitochondrial and nuclear sequence diversity should both be low.

Despite this large body of modelling generating empirically testable predictions, it is still unclear whether CMS factors are maintained as polymorphisms for functionally different alleles within gynodioecious populations by long-term balancing selection, or predominantly experience arms races. These different outcomes should be empirically distinguishable by analyses of sequences of the genes involved, and closely linked genome regions. Observing the footprint of a selective sweep would imply a recent spread of a cytoplasmic genotype throughout the population; a similar footprint would be expected after either spread of an advantageous haplotype in an arms race event, or loss of cytoplasmic polymorphism in a metapopulation, as in Dufay & Pannell's (2010) model. Such events can therefore be detected from low sequence diversity (reviewed in Tellier, Moreno-

Gámez, & Stephan, 2014), in a genome region, whose size will depend on the recombination frequency in the region (Kim & Stephan, 2002). In contrast, long-term balancing selection can lead to functionally different alleles being associated with neutral variants, forming extended haplotypes that may sometimes be shared between closely related species (trans-specific polymorphisms). This requires the maintenance of different alleles for long enough for new neutral mutations to arise, and recombination infrequent enough to allow them to remain associated with different selected alleles (Charlesworth, 2006). Trench warfare situations may often not persist for long enough, or recombination may occur too often, to produce a footprint of balancing selection (Tellier et al., 2014). Moreover, like host-pathogen systems (Tellier & Brown, 2007), CMS systems may undergo cyclical allele frequency changes (see above). If very high or low frequencies occur in finite populations, the functional polymorphism can be lost and the commoner allele fixed, making the situation resemble an arms race (Tellier et al., 2014).

Sequence-based tests between arms races and long-term maintenance of mitochondrial diversity have been attempted in various gynodioecious species, assuming that CMS factors are located in the mitochondrial genome, as in the crop plants so far studied (Chase, 2007; Hanson & Bentolila, 2004), and in *S. vulgaris*, based on genetic analyses (Charlesworth & Laporte, 1998). Overall, the tests, in the genus *Silene* do not conclusively support either long-term balancing selection or arms races (Supplementary Table 1). Some studies did not estimate divergence from outgroup species, so high mutation rates could not be excluded. Furthermore, organelle genomes also show greater subdivision than nuclear genes, and thus increased intraspecific diversity (Wakeley, 1999), and only one study has corrected for this effect, concluding that cytoplasmic diversity was no higher than expected from this effect alone (Ingvarsson, 2004). Moreover, recombination between mitochondrial genes is documented in heteroplasmic individuals of *S. vulgaris*, which are not extremely rare in natural populations (as reviewed by McCauley, 2013). Frequent recombination will eliminate associations between sequence variants and CMS factors maintained by balancing selection, making any form of selection undetectable, except in regions very close to the genes under selection. It is therefore important to study diversity of many mitochondrial genes, as different genes could suggest different conclusions.

In *Lobelia siphilitica*, another gynodioecious species with nucleo-cytoplasmic inheritance (Dudle, Mutikainen, & Delph, 2001), haplotype diversity for the mitochondrial *cob* and *cox1* genes was low, but higher than in the hermaphroditic *L. cardinalis*, and diversity within populations correlated positively with female frequencies (Delph & Montgomery, 2014), suggesting that mitochondrial haplotypes might be associated with male-sterility factors maintained by balancing

selection. However, it is difficult to exclude the possibility that lower diversity in hermaphroditic than gynodioecious populations is due to their having higher self-fertilization rates, since high self-fertilization rates correlate with low sequence diversity (reviewed by Charlesworth, 2003).

Achieving a complete understanding of gynodioecious systems will require the frequencies of CMS and restorer alleles to be known (Olson & McCauley, 2002), which is very difficult. The genes involved in causing male sterility are still known in only a few wild species, mainly close relatives of crops (e.g. Yamamoto et al., 2008). Molecular markers in restorer genes are not available in natural gynodioecious plant populations, and frequencies of a gene known to cause male sterility have so far been estimated in only one gynodioecious plant, the Japanese radish*, Raphanus sativus* (Brassicaceae), whose mitochondrial *orf138* gene is the Ogura radish cytotype's CMS factor; the frequency of an *orf138* variant correlates with the frequency of females in natural *R. sativus* populations (Murayama, Yahara, & Terachi, 2004). In the wild relative of sugar beet*, Beta vulgaris* ssp. *maritima*, at least four of 20 mitochondrial restriction fragment length "cytotypes" were found in male-sterile plants, and are associated, though not completely, with specific CMS types (Cuguen et al., 1994; Dufay, Cuguen, Arnaud, & Touzet, 2009). In sugar beet, *orf129* is responsible for male-sterility in two sterilizing cytoplasms in wild beet from Pakistan (Yamamoto et al., 2008), but its frequency has not been estimated. Frequencies of three "CMS-related cytotypes" have been studied in wild beet populations from the coasts of Normandy (France) and nearby islands (Dufay et al., 2009), but beet mitochondrial sequences have not been analysed to test for balancing selection.

Here, we used *P. lanceolata*, a widespread gynodioecious species free from some complications that make it difficult to understand the maintenance of females. First, self-incompatibility ensures that populations do not differ in their selfing rates. This species has nucleo-cytoplasmic genetic control of male-sterility (see above). The model studied by Couvet *et al*. (1998) should thus apply. Second, the relative female reproductive fitnesses of females and hermaphrodites should not depend on pollinator service, as the species is wind pollinated. Gynodioecy appears to be old-established in the genus *Plantago*, and female plants are found in 90% of northern European species (e.g. Van Damme, Hundscheid, Ivanovic, & Koelewijn, 2004; Van Damme, 1982), from several subgenera (Rønsted, Chase, Albach, & Bello, 2002).

The present study tests for an excess of mitochondrial sequence diversity within *P. lanceolata*, using the HKA test (Hudson, Kreitman, & Aguadé, 1987) with an outgroup species to allow for potential different nuclear and mitochondrial gene mutation rates. Such tests are essential when comparing nucleotide diversity estimates in nuclear and organelle genomes in the genus *Plantago*, because some lineages have exceptionally high synonymous site mitochondrial substitution rates,

sometimes exceeding the average for angiosperm nuclear genes (D. Sloan, Oxelman, Rautenberg, & Taylor, 2009), which is generally about 10 times higher than for mitochondrial genes (Drouin, Daoud, & Xia, 2008; Gaut, 1998; Wolfe, Li, & Sharp, 1987).

Methods

Plant species and populations studied

P. lanceolata plants were sampled from 15 geographically separated European natural populations (Figure 1) whose details were published already (Table 1 of Levsen, Bergero, Charlesworth, & Wolff, 2016) ; Supplementary Figure S1 and Table S2 list the individuals, including a few not previously included. Male-sterile frequencies are low (around 5%) in most *P. lanceolata* populations (Van Damme & VanDelden, 1982), and rarely exceeded 30% in the populations studied here (Supplementary Figure S2). We determined the sex phenotypes of as many individuals as possible in our samples, and classified females into the three phenotypes described by van Damme (1983), MS1, 2 and 3; these are shown in Supplementary Figure 1).

As outgroup species for our molecular evolutionary analyses (see below), we included *P. nivalis*, an hermaphroditic species from the Sierra Nevada in Spain (individuals provided by the Royal Botanic Garden, Edinburgh), and two species placed in the same subgenus (called *Albicans* or *Psyllium*) as *P. lanceolata* based on sequence divergence estimated from nuclear ITS and chloroplast *trnL* sequences (Rønsted et al., 2002), *P. lagopus*, a gynodioecious species, and a more distant outgroup, *P. patagonica*, a highly inbreeding hermaphroditic species (Sharma, Koul, & Koul, 1992).

Genes and sequencing

Eleven nuclear genes were chosen from a *P. lanceolata* transcriptome generated by 454 sequencing. Primers were designed for PCR and sequencing of partial sequences of 12 mitochondrial genes, based on partial mitochondrial genome assemblies from Illumina paired-end reads (N. Levsen and K. Wolff, unpublished). Supplementary Table S3 gives the primer sequences and lengths of the nuclear and mitochondrial regions analysed, with the estimated divergence values from several other *Plantago* species as well as from *Mimulus guttatus*. Three mitochondrial genes, ATP4, NAD6 and SDH4 had no variants within *P. lanceolata.*

Molecular evolutionary analyses

Concatenated sequences of the mitochondrial genes were generated (a total of 7,165 bp from the 9 mitochondrial genes in Supplementary Figure S1 that included at least one variant; the three genes without variants are noted in Supplementary Table S3) in order to determine the haplotypes present in the different *P. lanceolata* natural populations. After excluding all 16 individuals with heteroplasmy likely in any gene (Levsen et al., 2016) (Supplementary Figure S1), sub-sets of these sequences with genotypes determined for most sites (see details below) were used to analyse linkage disequilibrium (LD) between variants in different mitochondrial genes, using DnaSP, and to generate a network illustrating the relationships between the haplotypes detected (Bandelt, Forster, & Röhl, 1999); the network analysis excluded some genes where individuals had missing information.

Diversity (including π and θ values) and divergence were estimated using DnaSP (Librado & Rozas, 2009). We used silent site divergence for nuclear genes and synonymous site divergence for the mitochondrial sequences, which were almost exclusively coding regions (Supplementary Table S3). We denote both by K_S, and the mitochondrial divergence values suggested that *P. nivalis* or P. *lagopus* are the most suitable as close outgroup for testing whether mitochondrial diversity is unexpectedly high or low (see below).

To test for an excess of mitochondrial diversity within *P. lanceolata*, we used the maximum likelihood HKA test (MLHKA) implemented by Wright and Charlesworth (2004). We estimated nucleotide diversity values from the *P. lanceolata* sample for all site types, for each mitochondrial and nuclear gene sequenced, including loci with no polymorphism, and divergence values from the outgroup species for all site types. Effective population sizes (N_e values) differ for mitochondrial and nuclear sequences because the former are haploid and, assuming strict maternal inheritance, move only by seed dispersal, while the latter are diploid, and dispersed in both seeds and pollen (Hu & Ennos, 1999; Laporte & Charlesworth, 2002). Within-population diversity is consequently often lower for mitochondrial relative to nuclear sequences to an even greater extent than expected from the lower mitochondrial mutation rate that is documented in many plants (Wolfe et al., 1987), and subdivision, measured by F_{ST}, is generally high (Ingvarsson, 2004). In addition, the absence of paternal copies reduces the mitochondrial N_e value to half that for nuclear sequences in hermaphrodite populations, and it is further halved in dioecious populations if only females transmit mitochondria. However, in gynodioecious *P. lanceolata* maternally transmitted mitochondria can be

transmitted by both hermaphrodites and females, so its mitochondrial genome may have an N_e value greater than ¼ of that for nuclear genes.

To compare population subdivision for nuclear and mitochondrial genes, we estimated K_{ST} values using DnaSP (Librado & Rozas, 2009). K_{ST} is a measure similar to F_{ST}, but based on frequency differences at individual nucleotide sites (Hudson, Boos, & Kaplan, 1992). The significance of the K_{ST} values was tested using the K^*_{ST} and *Snn* statistics, as recommended (Hudson et al., 1992). K_{ST} estimates for individual genes are given in Supplementary Table 4. However, since the mitochondrial genes include few variants and, as documented below, probably recombine very rarely (Levsen et al., 2016), the K_{ST} values discussed below were computed using concatenated sequences of all the genes.

To test for a prolonged arms race scenario involving multiple functional substitutions, we did McDonald-Kreitman tests (McDonald & Kreitman, 1991). The arms race hypothesis predicts an excess of nonsynonymous over synonymous substitutions since divergence from *P. nivalis*, compared with the relative numbers of each type of variant among polymorphisms within *P. lanceolata*. We did not test whether more substitutions have occurred in the branch leading to the gynodioecious species, *P. lanceolata*, compared with the branch leading to the hermaphrodite, *P. nivalis*. This requires an outgroup to both these species, which is unfortunately not available. *P. patagonica* is unsuitable, because its mitochondrial sequence divergence from *P. lanceolata* is no higher than that from *P. nivalis*, and nor is *M. guttatus*, whose synonymous site divergence from *P. lanceolata* is too large for this analysis (Supplementary Table S3).

Results

Estimated mitochondrial mutation rate relative to the rate for nuclear genes, and choice of the outgroup species for HKA tests and for inferring ancestral states of variants in mitochondrial sequences

Sequences were obtained from *P. lanceolata* for a set of 12 nuclear and 13 mitochondrial genes (Supplementary Table S3). Divergence estimates from as many of these genes as possible from three other *Plantago* species, *P. nivalis*, *P. lagopus*, *P. patagonica*, were used to choose the outgroup species for further analyses. Based on the mitochondrial synonymous site divergence (K_S) estimates

(Supplementary Table S3), *P. nivalis*, with a mean value for mitochondrial genes of 0.097, appeared the most suitable as our outgroup; *P. lagopus* is too little diverged, and *P. patagonica* has a very similar K_S value to that for *P. nivalis*, but fewer gene sequences were available.

Plant mitochondrial genes generally have mutation rates about 10 times lower than nuclear genes (Gaut, 1998; Wolfe et al., 1987). Given the suggestion that *Plantago* species may have high rates for mitochondrial genes (see Introduction), we estimated the relative mutation rates using the *P. nivalis* data, and indeed found an unusually high mitochondrial rate, with a mitochondrial/nuclear ratio of silent site divergence of 0.73 (Supplementary Figure S3).

Mitochondrial haplotypes within P. lanceolata

To examine mitochondrial sequence diversity, we concatenated sequences of all 9 mitochondrial genes with sequence variants present in non-heteroplasmic *P. lanceolata* individuals (Supplementary Figure S1). This revealed two major haplotypes (Figure 2), which differ by fixed differences at seven sites in almost complete linkage disequilibrium (Figure 3). As previously inferred based on variants in two genes, *atp6* and *rps12* (following Levsen et al., 2016), one haplotype, which we call haplotype 1, was found in all 21 populations studied (Figure S1), while the other (haplotype 2) was found only in the populations from north east England and Scotland (Figure 1), which we refer to as NES populations (following Levsen et al., 2016). Based on 120 sequences (all sequences that included all 7 sites with fixed differences between the two haplotype classes, plus 6 sequences with information from 6 of these sites), the NES individuals included nearly equal numbers of both types (29 with haplotype 1, and 27 with haplotype 2), and both types were found in almost all these populations (Figure 1). If we include individuals with sites without genotype information (which should be reliable, given the strong linkage disequilibrium), all seven NES populations appear to include both types. In contrast, based on information from 70 individuals from 11 populations from the non-NES geographic region, haplotype 1 is fixed in these populations, and haplotype 2 is absent.

The strong linkage disequilibrium between all pairs of genes that included variants (Figure 3B) suggests that recombination is rare. Nevertheless, 14 of the 126 plants with genotypes for at least 6 of the SNPs that are diagnostic of the major haplotype have potentially recombinant haplotypes (Figure S1), consistent with our previous evidence for recombination (Levsen et al., 2016). Inter-haplotype recombination suggests that haplotypes 1 and 2 may have coexisted within

the three NES populations where they were found; one apparently recombinant individual was found in each of two populations (from Italy and Sweden), and, in the sample from Iceland, 9 of the 12 individuals are potential recombinants at position 1600 (Figure S1). Alternatively, however, given the high mitochondrial mutation rate, these apparent recombinant individuals could represent mutations to variants normally seen only in haplotype 2.

Diversity is low for the mitochondrial sequences (Table 1), but variants are present, particularly within the geographically widespread mitochondrial haplotype 1 (Figure 2), and 17 sites have non-singleton variants, several of them shared between geographically widely separated source populations, suggesting that this haplotype did not arise very recently. Haplotype 2 has lower diversity (with no non-singleton variants among 25 plants sequenced for all the genes, and no other variants among the 12 further individuals with incomplete information, see Supplementary Figure S1). This haplotype, therefore probably has a more recent common ancestor than haplotype 1, suggesting that it has spread recently through the populations in Northern England and Scotland.

Despite the presence of the two diverged haplotypes, overall nucleotide diversity was 25 times lower in the mitochondrial than the nuclear genes, with silent site π values of 0.00045 and 0.0114, respectively, excluding one nuclear gene, *cl51*, with extraordinarily high diversity (7.9%, see Table 1) and a significantly positive Tajima's D value that may indicate balancing selection (presence of paralogues is unlikely, as homozygotes were common, as well as heterozygotes). Lower mitochondrial diversity is predicted, given that the effective population size should be lower than that for nuclear genes, potentially accounting for diversity as much as fourfold lower than for nuclear sequences, though probably somewhat less (see above). The mutation rate difference we estimate (see above) can account for a further effect of at most twofold. The mitochondrial diversity is therefore certainly not higher than expected, and does not suggest long-term balancing selection.

This conclusion is supported by MLHKA tests comparing the diversity of mitochondrial genes with expected values based on data from nuclear genes, taking account of the effective population size difference, and using divergence from the *P. nivalis* sequences to correct for possible mutation rate differences between nuclear and mitochondrial sequences. The results were significant for a concatenation of all 12 mitochondrial (including three without variants shown in Supplementary Table S3); assuming that the mitochondrial genome's N_e value is 0.25 that for nuclear genes, the P value is 0.0091, and it is 0.0004 assuming a $N_{e,\ mitochondrial}/N_{e,\ nuclear}$ ratio of 0.5). Significant HKA test results were obtained for several individual mitochondrial genes (not shown), but none of them

suggests balancing selection. The genes with the highest diversity (*atp6* and *rps12*) did not yield significant results, and the significant genes have lower diversity than the others. The results therefore exclude the possibility that certain loci experience balancing selection, with recombination allowing other loci to have lower diversity. Instead they suggest that the entire mitochondrial genome has low diversity, and may have undergone selective sweep events recently enough that diversity has been reduced and has not yet recovered to its equilibrium level expected from its mutation rate.

Tajima's D values can be used to test for a recent selective sweep, which predicts negative values, and Table 1 shows such a tendency in the mitochondrial genes. However, negative D values could also arise from a recent expansion of the *P. lanceolata* population as a whole. If this is the cause of the overall negative D values in mitochondrial genes, it should then be found for nuclear sequences, and this was indeed seen (Table 1), though no locus in either genome had a significantly negative value; as mentioned above, the nuclear *cl51* gene has a significantly positive D value, which would obscure any signal of population expansion. Excluding this gene, the values are somewhat more negative for the mitochondrial genome (for silent sites, the median is -1.05 for the mitochondrial genes, versus -0.128 for nuclear ones), but not significantly so ($P = 0.13$ by a Wilcoxon-Mann-Whitney Rank Sum Test). This test therefore does not add any direct support for a selective sweep within the species.

Population subdivision for nuclear and mitochondrial genes, the age of the haplotypes, and the possibility of introgression

The HKA tests just described used sequences pooled across all the *P. lanceolata* populations sampled. As explained above, *P. lanceolata* exhibits regional differences in mitochondrial haplotypes, and strong subdivision is confirmed when these frequency differences are quantified using the K_{ST} statistic (see Methods and Table 1). Mitochondrial sequences' smaller N_e values than nuclear genes, and gene flow between populations mainly through seed dispersal, rather than through both seeds and pollen flow (Hu & Ennos, 1999; Ingvarsson, 2004), create higher K_{ST} values. Higher subdivision implies that mitochondrial sequences' coalescence times are higher than for nuclear genes, so an HKA test could falsely infer that diversity within the species as a whole is unexpectedly high, suggesting balancing selection (Ingvarsson, 2004). We did not apply Ingvarsson's suggested correction, because our conclusion is conservative that events reducing diversity, such as selective sweeps, and not balancing selection, have affected mitochondrial diversity.

Subdivision might, however, explain the presence of the two distinct haplotypes, as introgression into the NES populations might have occurred from an unsampled population that carries haplotype 2, for example, a separate race of *P. lanceolata* that has been isolated for long enough to acquire multiple substitutions in mitochondrial genes. The per nucleotide divergence between the *P. lanceolata* major haplotypes 1 and 2, based on substitutions at all site types, is small (0.0036, based on 63 haplotype 1 and 14 haplotype 2 sequences), but it does indeed exceed the within-haplotype nucleotide diversity (0.00136); using the sequences from individuals with no sites with missing sequence information, the inter-haplotype divergence is more than twice the diversity within haplotype (0.00103 and 0.00046, respectively). However, this diversity estimate is based on populations sampled from both the NES and non-NES regions, and therefore includes geographic differences among haplotype 1 sequences (see above). The divergence/diversity ratio is much higher if diversity is estimated from the NES samples alone.

The hypothesis of introgression predicts divergence in nuclear as well as mitochondrial sequences in the NES samples. However, comparison of nuclear sequence diversity between the NES and non-NES populations (Figure 4) does not support introgression. Higher values of three diversity measures were seen in the NES populations for only one nuclear gene, *agt1*, which shows no other signals of introgression, such as a high Tajima's D value (Table 1). Hybridisation of *P. lanceolata* with other species seems very unlikely, as none of the four other *Plantago* species in north-western Europe, *coronopus*, *maritima*, *major* and *media*, hybridizes with P. *lanceolata*. However, we cannot exclude the possibility that the presence of two haplotypes is might represent a history involving a population in the NES region that, during the last glacial period, contained *P. lanceolata* closely related to alpine individuals from other European regions.

We cannot conclusively exclude the possibility that haplotype 2 is maintained in other *P lanceolata* populations, but comparisons of diversity in the NES populations with that in other populations could potentially provide definitive evidence if enough nuclear as well as mitochondrial sequences were studied. It is, however, highly unlikely that haplotype 2 has been maintained at a low frequency in *P. lanceolata* for a long time, and recently spread to high frequencies in the NES populations (for example, because a non-sterility cytoplasm was favoured). Haplotype 2 sequences uniformly show much higher divergence from non-NES haplotype 1 sequences than is seen in inter-regional haplotype 1 comparisons. Inter-haplotype divergence therefore considerably pre-dates the inter-regional divergence, indicating a considerable time during which the polymorphism has been maintained.

Association between mitochondrial haplotypes and male sterility

The low mitochondrial diversity is not due to absence of male-sterility in the populations sampled (Supplementary Figure S2), nor to over-representation of populations without male-sterility (Supplementary Figure S1), and is consistent across our populations from which mitochondrial sequences were obtained from multiple plants (Supplementary Table 3). Variants of the *atp6* and *rps12* genes were previously shown to be associated with male sterility in the NES populations (Levsen et al., 2016). Our new results, including variants in several more mitochondrial genes, support this association, and show that, in the NES populations, where both haplotypes co-occur, haplotype 1 plants are much more likely to be female than haplotype 2 individuals (Table 2).

Molecular evolution of the P. lanceolata mitochondrial genome

In order to test further whether haplotype 2 arose more recently than haplotype 1, as suggested by the diversity results described above, we used the related species *P. nivalis* as an outgroup. The estimated divergence values between the *P. nivalis* sequence and the *P. lanceolata* haplotypes (including substitutions at all site types) differ strikingly: divergence per site is 0.015% for haplotype 2, versus only 0.004% for haplotype 1 suggesting that haplotype 1 may be an ancestral haplotype, whose sequence closely resembles that in *P. nivalis*, while haplotype 2 has multiple variants derived since the species split (Figure 3A).

We next tested whether the derived haplotype shows evidence of having evolved through a prolonged arms race involving multiple substitutions at functional sites, using McDonald-Kreitman tests (McDonald & Kreitman, 1991). The putatively derived haplotype 2 in *P. lanceolata* NES populations has 7 fixed differences from the inferred ancestral haplotype 1 sequence. Although non-synonymous polymorphisms maintained by balancing selection would be expected only in the genes responsible for CMS (which are not known in *P. lanceolata*), the lack of the footprint of long-term balancing selection suggests that mitochondrial genes undergo regular replacements by new alleles. Indeed, substitutions are found in several mitochondrial genes, and 6 of the 7 are nonsynonymous substitutions. These could be due to a low efficacy of purifying selection, or to adaptive changes in the mitochondrial genome. Despite the large divergence of the *P. nivalis* sequences, the MK test using the *P. nivalis* outgroup is marginally significant when only data from NES populations are included (Table 3), and is significant in the species as a whole when low frequency variants (< 5%) are excluded, to avoid including deleterious mutations. This suggests that the significant result could reflect adaptive non-synonymous changes in multiple mitochondrial genes. Derived amino acid

replacements may therefore be more abundant than expected in the haplotype associated with male-fertility in the NES populations, haplotype 2.

Discussion

Associations between P. lanceolata mitochondrial haplotypes and male-sterility

The male-sterility factors in naturally occurring gynodioecious populations have rarely been identified, despite several classical genetic studies demonstrating that cytoplasmic factors are involved (see, for example Andersson-Ceplitis & Bengtsson, 2002; Belhassen et al., 1991; Charlesworth & Laporte, 1998; Van Damme, 1983; Darracq et al., 2011; Dudle et al., 2001). With the ability to obtain sequences from individual plants, it has become possible to sequence multiple mitochondrial genes, enabling several informative analyses.

A first valuable result from our study is that high linkage disequilibrium demonstrates that recombination must be rare, justifying the assumption that sequencing mitochondrial genes, even without knowing which are involved in causing CMS, can reveal haplotypes that are associated with CMS mutations in natural populations, and allow reliable studies of such mutations' frequencies, as pioneered in *Beta vulgaris* (Dufay et al., 2009). Our results confirm that mitochondrial haplotype 1 is associated with male sterility, and haplotype 2 with hermaphroditism in the NES populations, where both haplotypes were found (Levsen et al., 2016).

In *P. lanceolata* the evidence for associations between the cytoplasmic factors causing male-sterility and mitochondrial sequences has until now been weak. Based on five populations studied in the Netherlands, the frequencies of plants with the MS1 male-sterility phenotype was strongly correlate with frequencies of the "CMSI" band pattern produced by restriction enzyme digestion; however, a sixth population with a lower MS1 frequency (Bm) had a higher CMSI, and the overall association was not strong (De Haan, Mateman, Van Dijk, & Van Damme, 1997). Moreover, three populations, Rei, Ven and Leek, with high frequencies of the MS2 sterility phenotype showed no correlation with the frequencies of the band patterns associated with MS2 sterility in genetic studies. Both this and our study, therefore, show associations between sex phenotypes and mitochondrial types in only some *P. lanceolata* populations.

It is puzzling, however, that the non-NES populations show no mitochondrial sequence variants. The presence of restorer alleles can produce populations in which some CMS factors cannot be detected genetically, using crosses (Charlesworth, 1981; Frank, 1989), but the absence of

mitochondrial variants is surprising. In our samples of both NES and non-NES populations, most females had the MS1 phenotype of van Damme (1983), with a few MS1-MS3 intermediates; only a single MS2 plant was observed (from an NES population Supplementary Figure 1). We did not attempt to correlate the frequencies of male-steriles and mitochondrial haplotypes, because we phenotyped few individuals per population (since our aim was to do HKA tests). However, although the non-NES populations (where our results suggest that gynodioecy could be under largely nuclear control) might be predicted to have generally low female frequencies, they do not differ as expected from the NES populations, where it is clearly cytonuclear (Levsen et al., 2016). The frequencies in our 22 plants sampled from two Netherlands populations (18 of which yielded sequence data, see Supplementary Figure S1) are higher than those previously reported, up to 20% (Figure 5 in De Haan, Luyten, et al., 1997), and a high frequency is seen in the Sussex sample (Figure S2).

These observations suggest the involvement of cytoplasmic factors previously demonstrated genetically in non-NES populations (De Haan, Luyten, et al., 1997), and are consistent with gynodioecy in these populations not being under wholly nuclear control.

P. lanceolata probably represents a metapopulation, which can produce joint cytonuclear polymorphism, with one maternal genotype undetectable in some demes, or else pure cytoplasmic polymorphism (Couvet et al., 1998), or purely nuclear nuclear control of femaleness (Dufay & Pannell, 2010). However, unless there is no cytoplasmic polymorphism, and male-sterility is controlled by variants at a nuclear restorer locus, sequencing should detect at least one polymorphism. The possibility that functional CMS variants in the non-coding sequences (most of the mitochondrial genome) were missed by our sequencing almost exclusively coding regions of mitochondrial genes seems to be ruled out by the observed strong genome-wide linkage disequilibrium (Figure 3). Possibly, however, gene conversion events might have occurred, affecting LD only very locally (Andolfatto & Nordborg, 1998), and generating an apparently invariant mitochondrial sequence across the sequenced regions, despite the presence of a sterility/non-sterility polymorphism at a site elsewhere in the mitochondrial genome. If so, detecting associations between mitochondrial genotypes and sex phenotypes will be difficult, even with sequence data. Alternatively, *P. lanceolata* populations may behave like the model in which cytoplasmic polymorphism was never maintained in the long term within sub-populations, which show purely nuclear control of femaleness (Dufay & Pannell, 2010).

Low mitochondrial nucleotide diversity but presence of two diverged haplotypes

The main goal of our study was to test whether the mitochondrial male-sterility types in *P lanceolata* have been maintained for long evolutionary times under balancing selection, as has been suggested in gynodioecious *Silene* populations (see Introduction). Our HKA tests, how conclusively that mitochondrial nucleotide diversity is lower than expected, given a mutation rate only slightly lower than that of the nuclear genes, excluding very long-term maintenance of mitochondrial male-sterility types in *P lanceolata*. Instead, the test suggests a selective sweep associated with recent spread of the extant mitochondrial genotype, which is also consistent with the generally more negative Tajima's D values for mitochondrial than nuclear genes, since positive values are expected if haplotypes are maintained for long evolutionary times at intermediate frequencies. On the other hand, the multiple inter-haplotype sequence differences indicate that the two major haplotypes do not have a recent common ancestor, suggesting maintenance of both for a considerable time, as expected under balancing selection. The lack of positive Tajima's D values might then be due to a recent population expansion.

These apparently contradictory findings can, however, be reconciled if advantageous mitochondrial mutations have spread without becoming fixed, as occurs in several of the models of cytonuclear male-sterility systems outlined above, which can produce large frequency fluctuations during the approach to stable equilibrium frequencies, or even permanent cyclical behaviour (Charlesworth, 1981; Couvet et al., 1998; Delannay et al., 1981; Gouyon et al., 1991). How this affects the molecular evolutionary signals of selection has not been studied, but certainly haplotypes may be lost from populations by genetic drift during periods of low frequency. Models of the similar dynamics in host-pathogen systems with gene-for-gene (GFG) resistance and susceptibility, another situation involving conflict, have demonstrated that cyclical frequency changes may make it difficult to distinguish between balancing selection and selective sweeps (Tellier et al., 2014). This view of CMS systems may help to explain the varied conclusions from studies in the genus *Silene* outlined in the Introduction section above.

The alternative possibility of hybridisation of *P. lanceolata* with other species seems unlikely to explain the co-occurrence of haplotypes 1 and 2, as none of the four other *Plantago* species in north-western Europe, *coronopus*, *maritima*, *major* and *media*, hybridizes with P. *lanceolata*. Possibly the presence of two haplotypes in the NES region represents unsampled *P. lanceolata* populations with the H2 haplotype, perhaps living in alpine regions during the last glacial period (although no such anciently diverged populations of the species have yet been discovered). If they exist, the H1 haplotype could have come in by subsequent migration from populations like those

from the non-NES region sampled here. However, our nuclear diversity data (Figure 4) do not support introgression from a diverged population, suggesting that the phenomenon is specific to the mitochondrial genome.

Overall, therefore, our data suggest that events occurred in *P. lanceolata* that reduced mitochondrial diversity, but allowed different haplotypes to persist for long enough to accumulate differences, but with "soft sweeps" due to fluctuating haplotype frequencies accounting for low within-haplotype variation. A similar process has been proposed to explain data from fish major histocompatibility systems, where the evidence argues against long-term balancing selection, but also against selective sweeps causing fixation of alleles (Lighten et al., 2017).

The theoretical models that produce cyclical frequency changes also generate fluctuations in nuclear restorer allele frequencies. However, studies of nuclear sequences in gynodioecious populations are unlikely to detect such changes, because recombination will occur, and they will therefore be detectable only at the restorer loci themselves, and sites very closely linked to them. A sample of only a few nuclear genes, such as the sample studied here, is very unlikely to include such sites.

One nuclear gene, *cl51*, does show unusual characteristics, but its diversity is unusually high (see Results). This gene displays strong haplotype structure, with one haplotype confined to the Feltre (Italy) population and the two populations from Elba (one of which also includes a different *cl51* haplotype). The other three haplotypes are scattered across populations from different geographic regions, and show no regional subdivision, so there is no evidence suggesting any connection with the mitochondrial genotypes (Supplementary Figure S4).

The multiple non-synonymous substitutions in haplotype 2 also require explanation. These might have been driven by successive selective sweeps involving fixations of advantageous non-synonymous changes, though the MK test results (Table 3) do not strongly suggest positive selection. An alternative is fixation by genetic drift during periods of very low N_e. It is also possible that sequence changes occur at high rates in genotypes carrying mutator factors (Parkinson et al., 2005); haplotypes that spread in populations may therefore tend to have unusually high mitochondrial mutation rates. High rates are indeed observed in *P. lanceolata*. This hypothesis might explain the striking association between taxa with accelerated mitochondrial mutation rates and the propensity to be gynodioecious (*Silene*, *Plantago*, *Ajuga* and Geraniaceae, see Introduction). Intriguingly, two previous studies of gynodioecious species, in *Beta vulgaris* ssp. *maritima* (Darracq et al., 2011) and *Silene vulgaris* (D. B. Sloan, Mueller, McCauley, Taylor, & Storchova, 2012), also found one

mitochondrial haplotype that is highly diverged from an inferred ancestral one, suggesting that a factor common to different CMS systems is involved.

Finally, we note that we inferred non-synonymous substitutions without excluding sites with RNA editing (usually C —> U), which occurs in plant mitochondrial transcripts (Stone & Storchova, 2015; Takenaka, Zehrmann, Verbitskiy, Haertel, & Brennicke, 2013). Editing mainly affects non-synonymous sites in protein-coding regions (Lu, Szmidt, & Wang, 1998), although the relationship between the editing frequency and the substitution rate can be partially obscured by the presence of processed paralogs, and/or gene conversion involving a cDNA copy in lineages that have lost most of their edited sites (Cuenca, Petersen, Seberg, J Davis, & Stevenson, 2010; Stone & Storchova, 2015), as has occurred in *Silene* (D. B. Sloan, MacQueen, Alverson, Palmer, & Taylor, 2010). Of the variants that differentiate haplotypes 1 and 2, several have the base C in haplotype 1 and T in haplotype 2. Possibly therefore, at some of these sites, haplotype 1 sequences are edited, and substitutions changing haplotype 2 to the edited state (loss of editing) were favoured. If so, some of the substitutions are not truly non-synonymous changes. However, the conclusion remains that considerable evolutionary time would be required for their fixation.

An alternative approach to detecting balancing selection is to test for trans-specific polymorphisms shared between different gynodioecious species. We did not test for trans-specific polymorphisms in *P. nivalis* (an alpine which may be a self-fertilising hermaphrodite, in which variability may be low, though detailed data are not available). The finding of haplotype 1 in our limited *P. nivalis* sample therefore does not definitively demonstrate the absence of a version of haplotype 2 in this species too. However, haplotype 2 clearly arose much more recently than the *P. lanceolata*-*P. nivalis* split, excluding its very long-term maintenance. It would be interesting to examine polymorphisms in *P. lagopus*, which is more closely related to *P. lanceolata*, and is also gynodioecious (Sharma et al., 1992).

Conclusions

Clearly, even though we can reject a model of long-term maintenance of mitochondrial haplotypes in *P. lanceolata*, much remains to be understood about gynodioecious populations. Sequencing studies similar to the present one may illuminate the situation in other species. However, although it is now possible to detect associations between mitochondrial sequences and sterility genotypes, it will be important to use such information along with genetic work. Only when nuclear restorer genotypes are also known will it be possible to classify mitochondrial haplotypes into different

functional types. Some may be unable to cause male-sterility, while others can do so, conditional on their restorer genotypes. Such understanding should improve our ability to disentangle fluctuations in the frequencies of the largely non-recombining mitochondrial genomes from changes connected with the recent demographic history of the populations. However, as the example of *P. lanceolate* shows, if mitochondrial genotypes undergo pronounced frequency fluctuations, it may be difficult to distinguish whether these genomes are under long-term balancing selection or experience recurring arms races.

Another task for the future is more modelling work. Despite similarities between cytonuclear gynodioecy and GFG models of host-pathogen systems (Frank, 1993), there are several important differences that could affect the outcomes, so conclusions about gynodioecy will require new modelling using appropriate assumptions, and including neutral sites whose evolution can be followed in populations with CMS systems. Most importantly, monocyclic GFG systems always have an unstable equilibrium and generate arms races, but fixation of resistance is rare, and happens only when mutations to new functional alleles are rare, or in small populations and in the absence of infectivity and resistance costs. This differs from Frank's findings that fixation of nuclear restorers of male-sterility occurs frequently in CMS systems, which have no property corresponding to infectivity. Based on the CMS models so far published, fluctuations in haplotype frequencies seem likely to occur, and should reduce diversity, because variation in the sizes and contributions of demes in a subdivided population usually decreases the effective size below that of an otherwise comparable panmictic population, and can greatly reduce diversity (Barton, 2000; Whitlock & Barton, 1997). One would therefore predict low diversity within each functionally different mitochondrial type, as we find in *P. lanceolata*.

Acknowledgments: This work was supported by the Natural Environment Research Council (grant number /J011444/1). DNA sequencing was carried out at Edinburgh Genomics.

Data Accessibility: The sequence alignments have been deposited in Dryad (doi:10.5061/dryad.th757vg).

Author Contributions: DC and KW conceived the study, RB performed the re-sequencing, DC and RB performed the population genetic analyses, NL and KW performed the mitochondrial genome sequencing. DC wrote the manuscript.

References

Andersson-Ceplitis, H., & Bengtsson, B. O. (2002). Transmission rates and phenotypic effects of mitochondrial plasmids and cytotypes in *Silene vulgaris*. *Evolution, 56*, 1586-1591.

Andolfatto, P., & Nordborg, M. (1998). The effect of gene conversion on intralocus associations. *Genetics, 148*, 1397-1399.

Baker, H. G. (1963). Evolutionary mechanisms in pollination biology. *Science, 139*, 877-883. doi:10.1126/science.139.3558.877

Bandelt, H.-J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution, 16*, 37-48.

Bartlett, H. (1911). On gynodioecism in *Plantago lanceolata*. *Rhodora, 13*, 199-206.

Barton, N. H. (2000). Genetic hitchhiking. *Phil. Trans. Roy. Soc. Lond. B., 355*, 1553-1562. doi:10.1098/rstb.2000.0716

Belhassen, E., Dommée, B., Atlan, A., Gouyon, P.-H., Pomente, D., Assouad, M. W., & Couvet, D. (1991). Complex determination of male sterility in *Thymus vulgaris* : genetic and molecular analysis. *Theoret. Appl. Genet., 82*, 137-143.

Charlesworth, D. (1981). A further study of the problem of the maintenance of females in gynodioecious species. *Heredity, 46*, 27-39.

Charlesworth, D. (2003). Effects of inbreeding on the genetic diversity of plant populations. *Phil. Trans. Roy. Soc. B, 358*, 1051-1070.

Charlesworth, D. (2006). Balancing selection and its effects on sequences in nearby genome regions. *PLoS Genetics, 2*, e64. doi: 10.1371/journal.pgen.0020064

Charlesworth, D., & Laporte, V. (1998). The male-sterility polymorphism of *Silene vulgaris*. Analysis of genetic data from two populations, and comparison with *Thymus vulgaris*. *Genetics, 150*, 1267-1282.

Chase, C. (2007). Cytoplasmic male sterility: a window to the world of plant mitochondrial-nuclear interactions. *Trends in Genetics, 23*, 81-90.

Couvet, D., Ronce, O., & Gliddon, C. (1998). The maintenance of nucleocytoplasmic polymorphism in a metapopulation: the case of gynodioecy. *American Naturalist, 152*, 59-70. doi:10.1086/286149

Cuenca, A., Petersen, G., Seberg, O., J Davis, & Stevenson, D. (2010). Are substitution rates and RNA editing correlated? *BMC Evolutionary Biology, 10*, 349. doi:10.1186/1471-2148-10-349

Cuguen, J., Wattier, R., Saumitou-Laprade, P., Forcioli, D., Mörchen, M., van-Dijk, H., & Vernet, P. (1994). Gynodioecy and mitochondrial DNA polymorphism in natural populations pf *Beta vulgaris* ssp *maritima*. *Gen. Sel. Evol., 26*, 87s-101s.

Darracq, A., Varre, J., Marechal-Drouard, L., Courseaux, A., Castric, V., Saumitou-Laprade, P., . . . Touzet, P. (2011). Structural and content diversity of mitochondrial genome in beet: a comparative genomic analysis *Genome Biology and Evolution, 3*, 723-736. doi:10.1093/gbe/evr042

De Haan, A. A., Mateman, A. C., Van Dijk, P. K., & Van Damme, J. M. M. (1997). New CMS types in *Plantago lanceolata* and their relatedness. *Theoretical and Applied Genetics, 94*, 539-548.

De Haan, A. A., Luyten, R.M.J.M., Bakx-Schotman, T.J.M.T. & Van Damme, J. M. M. (1997). The dynamics of gynodioecy in *Plantago lanceolata* . L. I. Frequencies of male-steriles and their cytoplasmic male sterility types. *Heredity, 79*, 453-462. doi:10.1038/hdy.1997.184

Delannay, X., Gouyon, P.-H., & Valdeyron, G. (1981). Mathematical study of the evolution of gynodioecy with cytoplasmic inheritance under the effect of a nuclear restorer gene. *Genetics, 99*, 169-181.

Delph, L., & Montgomery, B. (2014). The evolutionary dynamics of gynodioecy in *Lobelia*. *International Journal of Plant Sciences, 175*, 383-391. doi:10.1086/675572

Drouin, G., Daoud, H., & Xia, J. (2008). Relative rates of synonymous substitutions in the mitochondrial, chloroplast and nuclear genomes of seed plants. *Molecular Phylogenetics and Evolution, 49*, 827–831. doi:10.1016/j.ympev.2008.09.009

Dudle, D., Mutikainen, P., & Delph, L. (2001). Genetics of sex determination in the gynodioecious species *Lobelia siphilitica*: evidence from two populations. *Heredity, 86*, 265–276. doi:10.1046/j.1365-2540.2001.00833.x

Dufay, M., & Billard, E. (2012). How much better are females? The occurrence of female advantage, its proximal causes and its variation within and among gynodioecious species. *Annals of Botany, 109*(S1), 505-519. doi:10.1093/aob/mcr062

Dufay, M., Cuguen, J., Arnaud, J., & Touzet, P. (2009). Sex ratio variation among gynodioecious populations of sea beet: can it be explained by negative frequency-dependent selection? *Evolution, 63*, 1483–1497. doi:10.1111/j.1558-5646.2009.00653.x

Dufay, M., & Pannell, J. R. (2010). The effect of pollen versus seed flow on the maintenance of nuclear-cytoplasmic gynodioecy. *Evolution, 64*, 772-784. doi: 10.1111/j.1558-5646.2009.00847.x

Frank, S. A. (1989). The evolutionary dynamics of cytoplasmic male sterility. *American Naturalist, 133*, 345-576. doi:10.1086/284923

Frank, S. A. (1993). Coevolutionary genetics of plants and pathogens. *Evol. Ecol., 7*, 45-75.

Gaut, B. S. (1998). Molecular clocks and nucleotide substitution rates in higher plants. *Evolutionary Biology, 30*, 93-120.

Gouyon, P. H., Vichot, F., & Van Damme, J. (1991). Nuclear-cytoplasmic male sterility: single point equilibria versus limit cycles. *Amer. Nat., 137*, 498-514. doi:10.1086/285179

Hanson, M. R., & Bentolila, S. (2004). Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *The Plant Cell, 16*, S154 - S169.

Hu, X. S., & Ennos, R. A. (1999). Impacts of seed and pollen flow on population genetic structure for plant genomes with three contrasting modes of inheritance. *Genetics, 152*, 441-450.

Hudson, R. R., Boos, D. D., & Kaplan, N. L. (1992). A statistical test for detecting geographic subdivision. *Molecular Biology and Evolution, 9*, 138-151.

Hudson, R. R., Kreitman, M., & Aguadé, M. (1987). A test of neutral molecular evolution based on nucleotide data. *Genetics, 116*, 153-159.

Ingvarsson, P. K. (2004). Population subdivision and the Hudson–Kreitman–Aguade test: testing for deviations from the neutral model in organelle genomes. *Genetical Research (Cambridge), 83*, 31-39. doi:10.1017/S0016672303006529

Jacobs, M. S., & Wade, M. J. (2003). A synthetic review of the theory of gynodioecy. *American Naturalist, 161*, 837-851. doi:10.1086/375174

Kim, Y., & Stephan, W. (2002). Detecting a local signature of genetic hitchhiking along a recombining chromosome. *Genetics, 160*, 765-777.

Lahiani, E., Touzet, P., Billard, E., & Dufaÿ, M. (2015). Disentangling the effects of mating systems and mutation rates on cytoplamic diversity in gynodioecious *Silene nutans* and dioecious *Silene otites*. *Ecolgy and Evolution 5*, 1854-1862. doi:10.1002/ece3.1410

Laporte, V., & Charlesworth, B. (2002). Effective population size and population subdivision in demographically structured populations. *Genetics, 162*, 501-519.

Levsen, N., Bergero, R., Charlesworth, D., & Wolff, K. (2016). Frequent, geographically structured heteroplasmy in the mitochondria of a flowering plant, ribwort plantain (*Plantago lanceolata*). *Heredity, 117*, 1-7. doi:10.1038/hdy.2016.15

Lewis, D. (1941). Male sterility in natural populations of hermaphrodite plants. *New Phytologist, 40*, 56-63.

Li, W.-H. (1993). Unbiased estimation of the rates of synonymous and nonsynonymous substitution. *Journal of Molecular Evolution, 36*, 96-99.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*, 1451-1452.

Lighten, J., Papadopulos, A. S. T., Mohammed, R. S., Ward, B. J., Paterson, I. G., Baillie, L., . . . Oosterhout, C. v. (2017). Evolutionary genetics of immunological supertypes. *Nature Communications, 8*, 1294. doi:10.1038/s41467-017-01183-2

Lu, M. Z., Szmidt, A. E., & Wang, X. R. (1998). RNA editing in gymnosperms and its impact on the evolution of the mitochondrial *cox*I gene. *Plant Molecular Biology, 37*, 225-234.

McCauley, D. E. (2013). Paternal leakage, heteroplasmy, and the evolution of plant mitochondrial genomes. *New Phytologist*.

McCauley, D. E., & Olson, M. S. (2008). Do recent findings in plant mitochondrial molecular and population genetics have implications for the study of gynodioecy and cytonuclear conflict? *Evolution, 62*, 1013-1025. doi:10.1111/j.1558-5646.2008.00363.x

McDonald, J. H., & Kreitman, M. (1991). Accelerated protein evolution at the Adh locus in Drosophila. *Nature, 351*, 652-654.

Murayama, K., Yahara, T., & Terachi, T. (2004). Variation of female frequency and cytoplasmic male-sterility gene frequency among natural gynodioecious populations of wild radish (*Raphanus sativus* L.). *Molecular Ecology, 13*, 2459-2464. doi:10.1111/j.1365-294X.2004.02231.x

Olson, M. S., & McCauley, D. E. (2002). Mitochondrial DNA diversity, population structure, and gender association in the gynodioecious plant *Silene vulgaris*. *Evolution, 56*, 253–262.

Pamilo, P., & Bianchi, N. (1993). Evolution of the Zfx and Zfy, genes: Rates and interdependence between the genes. *Molecular Biology and Evolution, 10*, 271-281.

Parkinson, C., Mower, J., Qiu, J., Shirk, A., Song, K., Young, N., . . . Palmer, J. (2005). Multiple major increases and decreases in mitochondrial substitution rates in the plant family Geraniaceae. *BMC Evolutionary Biology, 5*, 73. doi:10.1186/1471-2148-5-73

Rønsted, N., Chase, M., Albach, D. C., & Bello, M. A. (2002). Phylogenetic relationships within Plantago (Plantaginaceae): Evidence from nuclear ribosomal ITS and plastid trnL-F sequence data. *Botanical Journal of the Linnean Society, 139*, 323–338. doi:10.1046/j.1095-8339.2002.00070.x

Ross, M. D. (1973). Inheritance of self-incompatibility in *Plantago lanceolata*. *Heredity, 30*, 169-176. doi:10.1038/hdy.1973.19

Sharma, N., Koul, P., & Koul, A. (1992). Genetic systems of six species of *Plantago* (Plantaginaceae). *Plant Systematics and Evolution, 181*, 1-9. doi:10.1007/BF00937582

Sloan, D., Oxelman, B., Rautenberg, A., & Taylor, D. (2009). Phylogenetic analysis of mitochondrial substitution rate variation in the angiosperm tribe Sileneae. *BMC Evolutionary Biology, 9*, 260. doi:doi:10.1186/1471-2148-9-260

Sloan, D. B., MacQueen, A. H., Alverson, A. J., Palmer, J. D., & Taylor, D. R. (2010). Extensive loss of RNA editing sites in rapidly evolving *Silene* mitochondrial genomes: selection vs.

retroprocessing as the driving force. *Genetics, 185*, 1369–1380. doi:10.1534/genetics.110.118000

Sloan, D. B., Mueller, K., McCauley, D., Taylor, D. R., & Storchova, H. (2012). Intraspecific variation in mitochondrial genome sequence, structure, and gene content in *Silene vulgaris*, an angiosperm with pervasive cytoplasmic male sterility. *New Phytologist, 196*, 1228-1239. doi:10.1111/j.1469-8137.2012.04340.x

Stone, J., & Storchova, H. (2015). The application of RNA-seq to the comprehensive analysis of plant mitochondrial transcriptomes. *Molecular Genetics and Genomics, 290*, 1-9. doi:10.1007/s00438-014-0905-6

Takenaka, M., Zehrmann, A., Verbitskiy, D., Haertel, B., & Brennicke, A. (2013). RNA editing in plants and its evolution. In *Annual Review of Genetics* (Vol. 47, pp. 335-352).

Tellier, A., & Brown, J. (2007). Stability of genetic polymorphism in host–parasite interactions. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 274*, 809-817. doi:DOI: 10.1098/rspb.2006.0281

Tellier, A., Moreno-Gámez, S., & Stephan, W. (2014). Speed of adaptation and genomic footprints of host-parasite coevolution under arms race and trench warfare dynamics. *Evolution, 68*, 2211-2224. doi:10.1111/evo.12427

Van Damme, J. (1982). Plantago, a multidisciplinary study. In P.J.C. Kuiper & M. Bos (Eds.), (pp. 241 – 249): Springer.

Van Damme, J. M. M. (1983). Gynodioecy in *Plantago lanceolata* L. II. Inheritance of three male sterility types. *Heredity, 50*, 253-273. doi:10.1038/hdy.1983.28

Van Damme, J., Hundscheid, M., Ivanovic, S., & Koelewijn, H. (2004). Multiple CMS-restorer gene polymorphism in *Plantago coronopus*. *Heredity, 93*, 175 – 181. doi:10.1038/sj.hdy.6800490

Van Damme, J. (1986). Gynodioecy in *Plantago lanceolata* L. V. Frequencies and spatial distribution of nuclear and cytoplasmic genes. *Heredity, 56*, 355-364. doi:10.1038/hdy.1986.57

Van Damme, J., & VanDelden, W. (1982). Gynodieocy in *Plantago lanceolata* L. I. Polymorphism for plasmon type. *Heredity, 49*, 303—318. doi:10.1038/hdy.1982.104

Wakeley, J. (1999). Nonequilibrium migration in human history. *Genetics, 153*, 1863-1871.

Whitlock, M. C., & Barton, N. H. (1997). The effective size of a subdivided population. *Genetics, 146*, 427-441.

Wolfe, K. H., Li, W.-H., & Sharp, P. M. (1987). Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proc. Natl. Acad. Sci. USA, 84*, 9054-9058.

Wright, S. I., & Charlesworth, B. (2004). The HKA test revisited: a maximum-likelihood-ratio test of the standard neutral model. *Genetics, 168*(October), 1071-1076.

Yamamoto, M., Shinada, H., Onodera, Y., Komaki, C., Mikami, T., & Kubo, T. (2008). A male sterility-associated mitochondrial protein in wild beets causes pollen disruption in transgenic plants. *The Plant Journal, 54*, 1027-1036. doi:10.1111/j.1365-313X.2008.03473.x

Figure legends

Figure 1. *P. lanceolata* populations studied. The locations are indicated by black dots for the NES populations, connected by arrows with pie charts showing the frequencies of the two major haplotypes described in the text (the key shows the colours denoting the two haplotypes), and by large pink dots for the non-NES populations, all of which have only haplotype 1. The sample sizes can be ascertained from Supplementary Figure S1).

Figure 2. Mitochondrial haplotype network for 94 sequences with 9 genes having at least one sequence variant in *P. lanceolate* (Supplementary Figure S1). Note that this concatenated sequence is 3,024 bp long, with 13 non-singleton segregating sites (shorter than the concatenation mentioned above, in order to include as many individuals as possible).

Figure 3. Haplotypes in the *P. lanceolata* mitochondrial genome, A. Summary of the variants defining haplotypes 1 and 2 (full details are shown in Supplementary Figure S1). B. Linkage disequilibrium based on pairwise D' for 13 SNPs found across 11 mitochondrial genes.

Figure 4. Testing for evidence of introgression using nuclear genes. The figure shows the results of comparing two sequence diversity measures (A) and haplotype diversity (B) with values in NES and non-NES populations. As far as possible, the comparison used populations from which both nuclear and mitochondrial sequences were available (Supplementary Table 4).

Supplementary figure legends and table titles

Figure S1. Variants in mitochondrial gene sequences from individuals from natural *P. lanceolata* populations and outgroup species, including *P. nivalis*. The figure also indicates the sexes of the individuals when these were ascertained. The different genes are indicated at the top, and the sites within them that showed variants in our samples, and whether the variants are synonymous or non-synonymous; singleton variants are also indicated.

Figure S2. Numbers of the different sex types in the populations studied where at least four plants, and estimated frequencies of male-steriles, including individuals with some male-sterile flowers, classified as intermediates (data from Figure S1).

Figure S3. A. Estimated mitochondrial gene sequence divergence between different potential outgroup species of *Plantago*, and nuclear gene divergence from the chosen outgroup, *P. nivalis*. Divergence was estimated per nucleotide site for the numbers of genes indicated for each species. Most sites were synonymous sites within coding sequences, but non-coding regions were present in one gene (*cox3*) and were included (see Supplementary Table S3), and therefore the y axis is labelled “silent site divergence”.

Figure S4. Neighbour-joining tree for the nuclear *Cl51* gene, estimated in MEGA using synonymous sites, and for all site types (only one non-synonymous variant was present in the sequenced region of this gene). The tree is rooted with *P. nivalis* as the outgroup, and Pamilo-Bianchi- Li correction for multiple substitutions was applied for synonymous sites (Li, 1993; Pamilo & Bianchi, 1993).

Table S1. Summary of studies in the genus *Silene* to test for long-term balancing selection versus epidemic dynamics.

Table S2. List of *P. lanceolata* populations sampled for the study.

Table S3. Genes sequenced, with the primers used and lengths sequences, and some divergence results. A: Mitochondrial genes, B: Nuclear genes. NOTE that this table has not yet been finalised.

Table S4. *P. lanceolata* samples used in the subdivision analyses and for the K_{ST} calculations shown in Table 1.

Table 1. Sequence diversity within *P. lanceolata*, and K_{ST} values for mitochondrial and nuclear genes in the same set of populations which yielded sequences for both (see Table S3), with *P* values for permutation tests of K^*_{ST} values (see Methods; unlike the mitochondrial genes, all nuclear genes sequenced included variants within *P. lanceolata*). Genes with subdivision significantly different from zero by this test (with $P < 0.05$) are shown in bold (non-significance is shown as "ns"). The results for nuclear genes are for silent sites (synonymous or non-coding), while those for mitochondrial genes are almost entirely for synonymous sites, because only *cox1* included non-coding sites; the row labelled "All genes" shows results for all mitochondrial genes, concatenated, including four mitochondrial genes with no silent site variants in *P. lanceolata* that are not included individually in the table (Table S2 gives the primers used).

Gene names	Length of sequence	Number of sequences	Number of silent sites	Silent or synonymous site diversity		Tajima's D values		Between NE and non-NES populations		Across populations with at least 2 alleles sequenced	
				π	θ	All sites	Silent sites	K_{ST}	P value	K_{ST}	P value
8 mitochondrial genes with variants in *P. lanceolata*											
atp6	660	42	156	0.015	0.003	-0.53	-1.11	**0.654**	0.001	**0.451**	ns
atp8	129	113	113	0.0049	0.006	-0.24	-0.77	**0.049**	0.009	**0.622**	ns
cox3 †	594	42	149.8	0.00033	0.0016	-1.12	-1.12	NA ‡	—	NA	—
cob	701	141	167.2	0.0004	0.0022	-0.15	-1.16	**0.381**	0.002	**0.558**	0.004
ccmfn	843	134	199.7	0.0002	0.0009	0.21	-0.82	**0.448**	0.012	**0.448**	ns
matr	790	119	195.4	0.00017	0.0019	-0.81	-1.53	**0.372**	0.007	**0.481**	0.029
mttb	533	157	126.9	0.0001	0.0014	-0.37	-0.98	**0.627**	0.006	**0.627**	0.044
rps12	60	147	14	0.031	0.012	1.49	1.49	**0.086**	ns	**0.779**	0.008
All genes	7,165	32	2,180	0.00045	0.00057	-0.14	-0.55	**0.583**	0	**0.820**	0

† Analysis using only a group of 41 long sequences from *P. lanceolata*

‡ Not analysed (few variants)

Table 1, continued

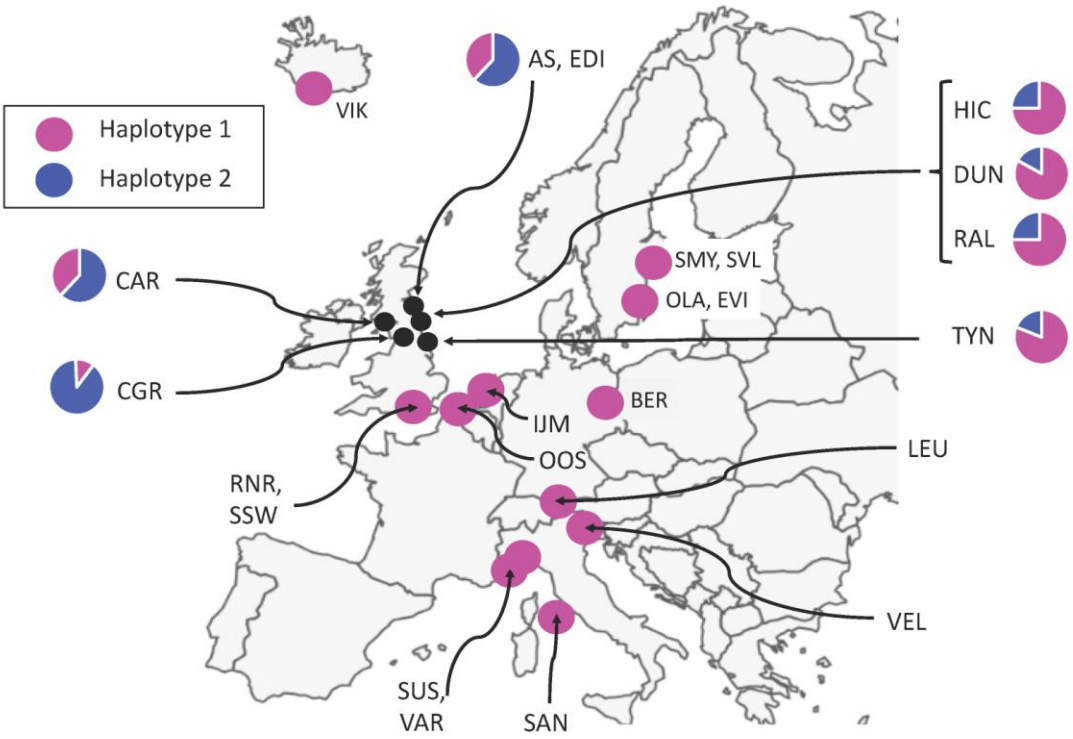
	Length of sequence	Length of sequence	Number of silent sites	Synonymous site diversity		Tajima's D values		Between NE and non-NES populations		Across populations with at least 2 alleles sequenced	
				π	θ	All sites	Silent sites	K_{ST}	P value	K_{ST}	P value
Nuclear genes											
acyb2	328	46	110	0.027	0.023	0.87	0.51	0.026	ns	0.086	0.033
aldh	476	32	353	0.006	0.008	-0.85	-0.77	< 0	—	0.142	ns
agt1	676	24	258	0.017	0.017	-0.155	-0.155	< 0	—	0.115	ns
ailp1	627	42	56	0.0026	0.002	0.13	0.62	< 0	—	**0.087**	0.035
cl51	558	46	126.7	0.079	0.041	2.51 ($P < 0.05$)	2.72 ($P < 0.01$)	< 0	—	**0.194**	0.005
ccr	895	44	147.1	0.005	0.008	-1.13	-1.09	< 0	—	**0.138**	0.009
fri	785	46	181.8	0.0011	0.0025	-0.18	-1.0	0.011	ns	**0.206**	0
psaf	605	30	171.8	0.029	0.025	-0.21	-0.10	**0.101**	0.001	**0.250**	0.007
psal	474	40	355	0.0056	0.0073	-0.16	0.29	**0.044**	0.019	**0.167**	0
rcc1	918	46	136.5	0.0058	0.0088	-1.28	-1.22	0.024	ns	**0.127**	0.034
tbl13	398	28	37.9	0.015	0.013	0.21	0.15	0.013	ns	**0.429**	0

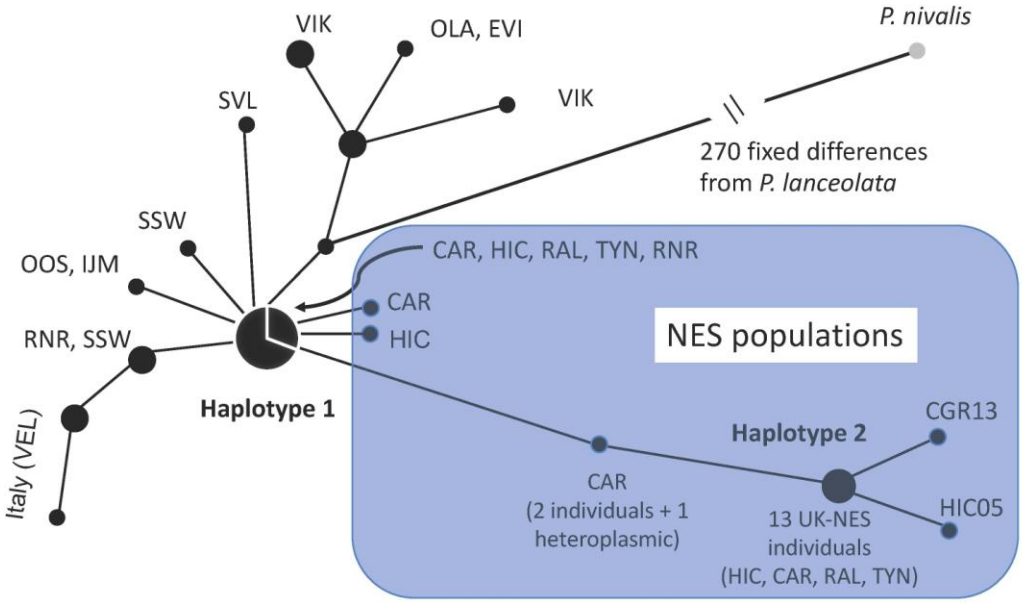
Table 2. Summary of the sex phenotypes of plants with different mitochondrial haplotypes. The results are based on 81 individuals where information was available for their sex type as well as the mitochondrial haplotypes (defined by SNPs). No haplotype 2 plants were found in the non-NES populations.

Population set or arrangement region	Mitochondrial haplotypes (their frequencies)	Numbers of each sex phenotype		P value, Fisher's exact test
		H (%)	Male-sterile	
Population set				
NES	Haplotype 1 (72%)	14 (41%)	20	0.0004
	Haplotype 2 (28%)	11 (85%)	2	
All populations pooled	Haplotype 1	35	33	0.0022
	Haplotype 2	11	2	

Table 3. McDonald-Kreitman test results for mitochondrial genes.

Comparison	Polymorphisms in *P. lanceolata*		Fixed differences from *P. nivalis*		P value
	Synonymous	Non-synonymous	Synonymous	Non-synonymous	
All 16 sites in the sequences with most of the 92 individuals genotyped	7	11	163	123	0.149
Excluding variants at frequencies < 5% in *P. lanceolata*	2	8	161	115	0.022
NES sequences only	1	6	160	114	0.045





A

P. lanceolata populations	Haplotype	Minimum numbers of individuals	Non-singleton variants in genes: atp6	cob		ccmfn	matr	matr	rps12
ALN	1	5	C	C	C	A	G	C	T
	2	1	T	T	T	C	—	T	C
CAR	1	1	C	C	C	A	G	C	T
	2	3	T	T	T	C	A	T	C
DUN	1	3	C	C	C	A	G	C	T
	2	1	T	T	T	C	A	T	C
HIC	1	5	C	C	C	A	G	C	T
	2	4	T	T	T	C	A	T	C
TYN	1	3	C	C	C	A	G	C	T
	2	2	T	T	T	C	A	T	C
AE, EDI	1	5	C	—	—	—	—	C	T
	2	6	T	T	T	—	—	T	C
CGR	2	11	T	T	T	C	A	T	C
Non-NES populations	1	56	C	C	C	A	G	T	T
Other species									
Mimulus guttatus			C	C	C	A	G	T	T
Plantago nivalis			C	C	C	A	G	C	T
Synonymous/non-synonymous substitutions			N	N	N	N	N	N	S

B